

# Extremely efficient cleavage of eIF4G by picornaviral proteinases L and 2A in vitro

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Received 8 May 2000; accepted 1 August 2000

Edited by Hans-Dieter Klenk

**Abstract** Certain picornaviruses encode proteinases which cleave the translation initiation factor eIF4G, a member of the eIF4F complex which recruits mRNA to the 40S ribosomal subunit during initiation of protein synthesis in eukaryotes. We have compared the efficiency of eIF4G cleavage in rabbit reticulocyte lysates during translation of mRNAs encoding the foot-and-mouth disease virus leader proteinase (L<sup>pro</sup>) or the human rhinovirus 2A<sup>pro</sup>. Under standard translation conditions, L<sup>pro</sup> cleaved 50% of eIF4G within 4 min after initiation of protein synthesis, whereas 2A<sup>pro</sup> required 15 min. At these times, the molar ratios of proteinase to eIF4G were 1:130 for L<sup>pro</sup> and 1:12 for 2A<sup>pro</sup>, indicating a much more efficient in vitro cleavage than previously observed. The molar ratios are similar to those observed during viral infection in vivo. © 2000 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

**Key words:** Foot-and-mouth disease L proteinase; Human rhinovirus 2A proteinase; Picornavirus; eIF4G cleavage; Inhibition of protein synthesis; Initiation of translation

## 1. Introduction

Members of the eIF4 (eukaryotic initiation factor) group of initiation factors play a central role in the initiation of protein synthesis in eukaryotes [1]. These factors comprise the cap binding protein eIF4E, the RNA helicase eIF4A, the RNA binding protein eIF4B and the adapter protein eIF4G. In mammalian systems, eIF4E, eIF4A and eIF4G can be isolated as a complex designated eIF4F. eIF4F, together with eIF4B and eIF3, recognise the cap structure at the 5' end of the cellular mRNA, unwind RNA secondary structure in an ATP-dependent manner and enable binding of the 40S ribosomal subunit to the mRNA [2–4].

The eIF4F complex is an important control point for the overall rate of protein synthesis [5]. During the replication of certain picornaviruses such as foot-and-mouth disease virus (FMDV), human rhino- (HRV) and entero-viruses, translation of capped cellular mRNA in the infected cell is shut down by specific cleavage of eIF4G [6–8]. Initiation of protein synthesis from viral mRNA is unaffected as it initiates internally and does not require a cap structure [9,10]. Substantial biochemical and genetic evidence shows that the papain-like leader proteinase (L<sup>pro</sup>) of FMDV and the chymotrypsin-like 2A proteinases (2A<sup>pro</sup>) of HRVs and enteroviruses cleave eIF4G [11–14]. Indeed, the cleavage sites of the enzymes on

eIF4G have been determined; they are separated by six amino acids [15,16]. Despite this knowledge, the mechanism of this cleavage is still in dispute. Using purified eIF4F and recombinant proteinases, several groups have demonstrated that eIF4G can be cleaved directly in vitro [12,14,16,17]. However, the proteinase concentration present in the infected cell at the time of eIF4G cleavage is much lower than the amount of proteinase required for eIF4G cleavage in vitro [13,18,19]. This has led to the suggestion that the picornaviral proteinases may activate latent cellular proteinases which then amplify the response [20,21]. This view has been strengthened, at least for poliovirus, by the observation that the majority of the eIF4G cleaving activity isolated from infected cells does not appear to co-purify with 2A<sup>pro</sup> containing fractions [22].

Based on the observations that HRV2 2A<sup>pro</sup> cleaves eIF4G bound to eIF4E more efficiently than eIF4G alone [17] and that most of the eIF4G in actively translating rabbit reticulocyte lysates (RRLs) is both complexed to eIF4E and bound to ribosomes [23], we reasoned that cleavage of eIF4G may occur during synthesis of the picornaviral proteinases. The spatial proximity of the nascent proteinase, which is synthesised on the ribosome, and eIF4G, which is bound to the ribosome via eIF3, could be beneficial to the cleavage reaction. To investigate this, we examined and quantified the cleavage of endogenous eIF4G in rabbit reticulocyte lysates during synthesis of picornaviral proteinases. We present here a study of the kinetics of eIF4G cleavage by FMDV L<sup>pro</sup> from serotype O1<sub>k</sub> and by HRV2 2A<sup>pro</sup>.

## 2. Materials and methods

### 2.1. Plasmids

The plasmid pet8cFMDV Lb<sup>pro</sup> (encoding the mature Lb<sup>pro</sup>) contains the FMDV nucleotides 892–1413 of the FMDV O1<sub>k</sub> cDNA [24] followed by two stop codons, cloned into the *NcoI* and *BamHI* restriction sites of the T7 polymerase expression vector pET8c (Novagen). The plasmid pet8cFMDV Lb<sup>pro</sup>VP4VP2 (encoding the mature Lb<sup>pro</sup>, all of VP4 and 78 amino acids of VP2) contains the FMDV nucleotides 892–1896 followed by two stop codons similarly cloned into pet8c [16]. In pet8cFMDV Lb<sup>pro</sup>C51A VP4VP2, the active site cysteine 51 is replaced by an alanine. The plasmid pet8cHRV2 2A<sup>pro</sup> has been previously described [12]. It contains the HRV2 nucleotides 3161–3586 encoding the mature 2A<sup>pro</sup> followed by two stop codons. Thus, all mRNAs produced from the above plasmids have the same sequence at the 5' untranslated region.

### 2.2. In vitro transcription and translation

Plasmids pet8cFMDV Lb<sup>pro</sup> and pet8cHRV2 2A<sup>pro</sup> (2–5 µg) were linearised with *BamHI*; plasmids pet8cFMDV Lb<sup>pro</sup>VP4VP2 and pet8cFMDV Lb<sup>pro</sup>C51A VP4VP2 were linearised with *SalI* (FMDV nucleotide 1712; see Fig. 2A). In vitro transcription was as described [12]. RNA concentrations were quantified by OD<sub>260</sub> measurement and confirmed by electrophoresis on a 1% agarose gel containing 0.1% sodium dodecyl sulfate.

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In vitro translation reactions (typically 50  $\mu$ l) contained 70% RRL (Promega), 21  $\mu$ Ci [ $^{35}$ S]Met/Cys label (1170 mCi/mmol, American Research Company), amino acids (except methionine) at 20  $\mu$ M and the RNA as indicated. After preincubation for 2 min at 30°C, translation was started by addition of RNA. Aliquots (10  $\mu$ l) were removed at the designated time points and the reaction was stopped by immediate transfer to ice, the addition of unlabelled methionine and cysteine to a final concentration of 2 mM and Laemmli sample buffer.

### 2.3. Electrophoresis and immunoblotting

The polyacrylamide gel electrophoresis system described by Dasso and Jackson [25] was used for analysis of both translation products and the state of eIF4G. Aliquots (2  $\mu$ l for translation products and 3  $\mu$ l for immunoblotting) were diluted with sample buffer and subjected to gel electrophoresis on 15% and 6% gels, respectively. The 15% gels were fluorographed by incubation for 30 min in 1 M sodium salicylate (dissolved in 45% methanol) and exposed after drying on Kodak Biomax MR film. Immunoblotting and detection of eIF4G with the anti-eIF4G peptide 7 antiserum [26] was as described [27], except that Immobilon-P membranes (Millipore) were used and that the second antibody (anti-rabbit IgG coupled to alkaline phosphatase) was from Sigma.

### 2.4. Quantification of protein synthesis

Radioactivity was counted in the dried polyacrylamide gels using an Instant Imager (Canberra Packard); the counting efficiency was determined to be 65% by counting known amounts of [ $^{35}$ S]Met/Cys label spotted onto a dried gel. The amounts of proteinase synthesised were calculated as follows (all amounts are given per  $\mu$ l of final translation reaction): 5 pmol unlabelled methionine (contributed by the endogenous methionine in the lysate itself, as stated by the manufacturer) and 22 pmol cysteine (2 pmol endogenous and 20 pmol from the amino acid mix) were present in the translation reaction. The addition of 0.42  $\mu$ Ci [ $^{35}$ S]Met/Cys label (70% methionine, 25% cysteine and 5% other) at 1170 Ci/mmol adds 0.25 pmol methionine and 0.09 pmol cysteine to the reaction. Thus, in 1  $\mu$ l translation reaction, 5.25 pmol of methionine and 22.09 pmol of cysteine were present. Taking into account the counting efficiency and the reference data, a total of 255 300 cpm were present per  $\mu$ l. Allowing for the distribution of the radioactivity between the methionine (75%) and cysteine (25%), 5.25 pmol methionine and 22.09 pmol of cysteine correspond to 191 500 and 63 800 cpm, respectively. The Lb<sup>pro</sup> has three methionines and two cysteines; as methionine is limiting, a maximum of 1.75 pmol (191 500 cpm) of Lb<sup>pro</sup> per  $\mu$ l can be synthesised. This amount of Lb<sup>pro</sup> contains 3.5 pmol of cysteine, corresponding to 10 100 cpm. Thus, 1.75 pmol Lb<sup>pro</sup> represents in total 201 600 cpm; the specific radioactivity of Lb<sup>pro</sup> is therefore 115 cpm/fmol (19.8 pg, MW Lb<sup>pro</sup> 19 800). For HRV2 2A<sup>pro</sup> (three methionines and seven cysteines), a maximum of 1.75 pmol/ $\mu$ l can also be synthesised, with concomitant incorporation of 12.25 pmol cysteine (35 400 cpm). Thus, 1.75 pmol HRV2 2A<sup>pro</sup> represent in total 226 900 cpm; the specific radioactivity of HRV2 2A<sup>pro</sup> is therefore 129 cpm/fmol (16.3 pg, MW 2A<sup>pro</sup> 16 300).

## 3. Results

### 3.1. Cleavage of eIF4G during in vitro protein synthesis of FMDV L<sup>pro</sup>

L<sup>pro</sup> is the first protein encoded on the FMDV genome. As protein synthesis initiates from one of two AUG codons, two forms of L<sup>pro</sup>, Lab<sup>pro</sup> and Lb<sup>pro</sup>, can be generated [28]. Both forms have the same enzymatic activities [29]. Based on evidence that the 28 amino acid shorter Lb<sup>pro</sup> form is the biologically relevant one [30], we have investigated this form.

Fig. 1A shows the synthesis of FMDV Lb<sup>pro</sup> in RRLs programmed with an uncapped mRNA encoding the mature proteinase followed by two stop codons. Synthesis of Lb<sup>pro</sup> was detectable after 4 min. The concentration of Lb<sup>pro</sup> reached (see Section 2) at a given time point is indicated underneath the respective lane (Fig. 1A).

To investigate the state of eIF4G in these reactions, ali-

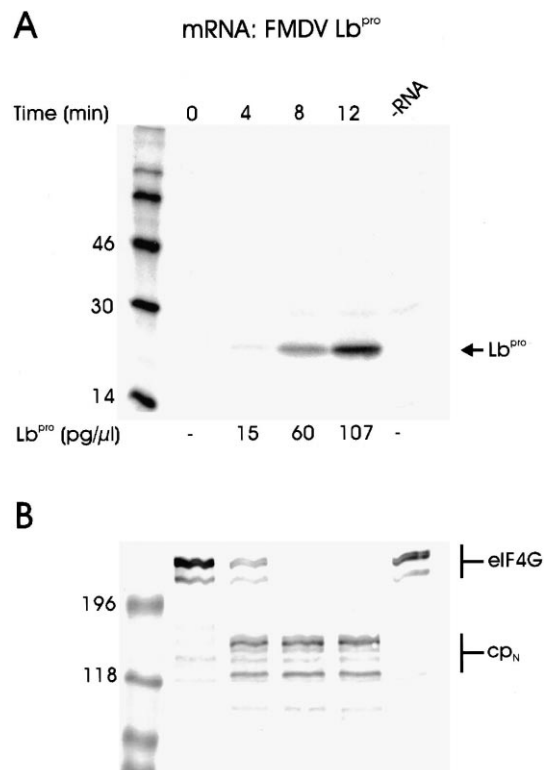


Fig. 1. Kinetics of eIF4G cleavage during in vitro translation of Lb<sup>pro</sup> from the mRNA FMDV Lb<sup>pro</sup>. RRLs were incubated with or without the uncapped mRNA FMDV Lb<sup>pro</sup> (7 ng/ $\mu$ l) as described in Section 2; protein synthesis was terminated at the times given by placing the samples on ice followed by the addition of unlabelled methionine and cysteine to 2 mM and Laemmli sample buffer. Aliquots (2 and 3  $\mu$ l, respectively) were analysed on 15% polyacrylamide gels followed by fluorography for the synthesis of Lb<sup>pro</sup> (A) and on 6% polyacrylamide gels followed by immunoblotting for the status of eIF4G (B). The position of the Lb<sup>pro</sup> is marked in A; the concentration (in pg/ $\mu$ l) of Lb<sup>pro</sup> reached at each time point is indicated underneath the lane. In B, intact eIF4G and the cleavage product cp<sub>N</sub> are marked. Protein standards (in kDa) are indicated in both panels.

quots of the translation mixture were blotted onto an Immobilon-P membrane and probed with an antiserum to the N-terminus of eIF4G. Intact eIF4G is observed as a series of bands with an apparent molecular weight around 220 kDa. Cleavage by the Lb<sup>pro</sup> gives rise to a similar series of bands migrating with an apparent molecular weight between 150 and 120 kDa (Fig. 1B), although the calculated molecular weight is only 80 kDa. These are the N-terminal cleavage products, designated cp<sub>N</sub> [12,15]. Clearly, the cleavage of eIF4G is extraordinarily efficient in this system. After 4 min, approximately 50% of the eIF4G had been cleaved; the concentration of Lb<sup>pro</sup> at this time had risen to 15 pg/ $\mu$ l (0.76 nM). Essentially complete eIF4G cleavage was observed after 8 min. No cleavage of the eIF4G was observed in lysates lacking RNA (Fig. 1B).

### 3.2. Cleavage of eIF4G during in vitro protein synthesis of FMDV L<sup>pro</sup> VP4VP2

To investigate whether similar kinetics of eIF4G cleavage are observed when Lb<sup>pro</sup> processes itself from the growing polypeptide chain as it does in vivo, we programmed RRLs

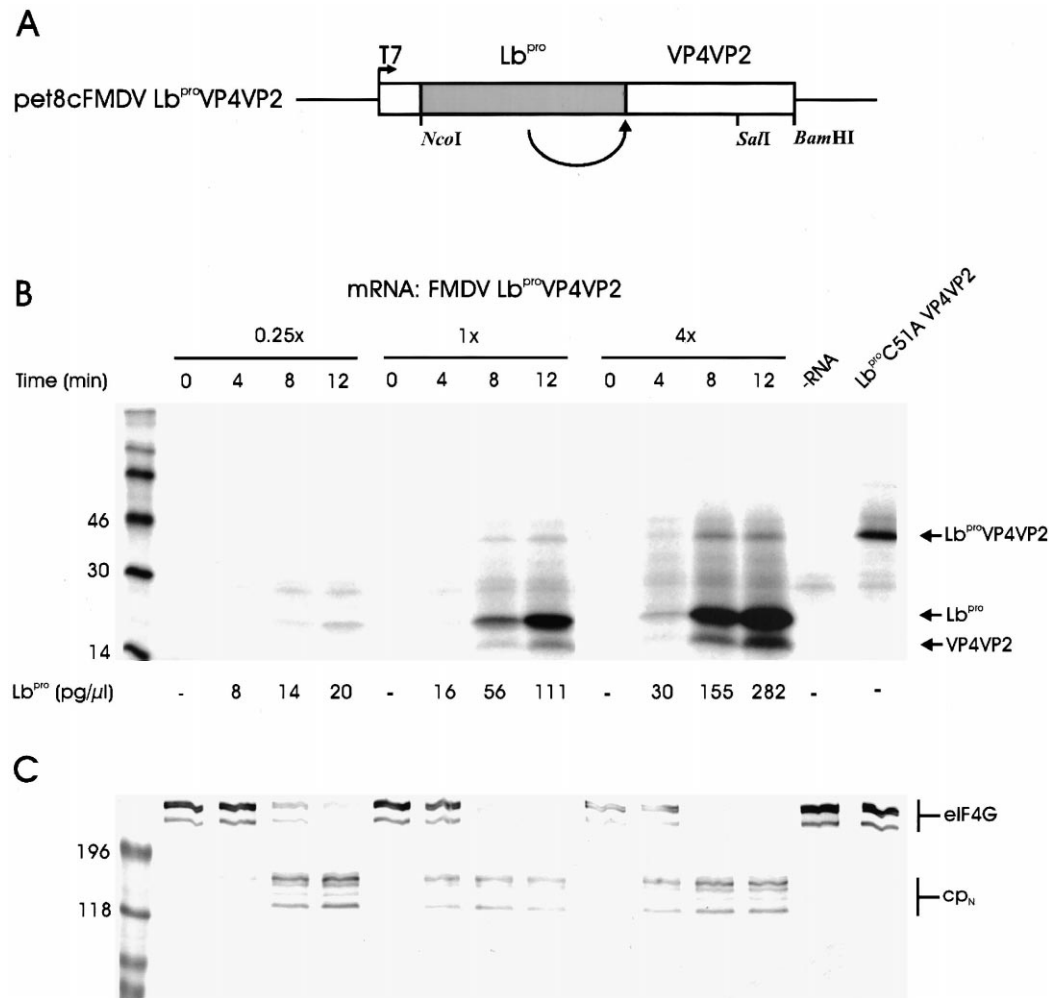


Fig. 2. Kinetics of eIF4G cleavage during in vitro translation of Lb<sup>pro</sup> from the mRNA FMDV Lb<sup>pro</sup>VP4VP2. The structure of the expression block of pet8cFMDV Lb<sup>pro</sup>VP4VP2 is shown in A. After cleavage with *SalI*, uncapped mRNA was transcribed and used at a concentration of 4.2 ng/μl to program protein synthesis (B, panel 1×) as described in Fig. 1. In addition, two further mRNA concentrations were tested, 1.1 ng/μl (B, panel 0.25×) and 16.8 ng/μl (B, panel 4×). The mRNA FMDV L<sup>pro</sup>C51A VP4VP2 (9 ng/μl) encoding the inactive Lb<sup>pro</sup>C51A VP4VP2 was also used to program RRLs. Analysis of the synthesis of Lb<sup>pro</sup> (B) and the cleavage of eIF4G (C) was as in Fig. 1. The positions of the Lb<sup>pro</sup>, the cleaved product VP4VP2 and the uncleaved L<sup>pro</sup>VP4VP2 are marked in B; the concentration (in pg/μl) of Lb<sup>pro</sup> reached at each time point is indicated underneath the lane. Note that in part B, the middle and right panels are overexposed in order to visualise the translation products in the left panel.

with uncapped RNA transcribed from pet8cFMDV Lb<sup>pro</sup>VP4VP2 cut with *SalI* (Fig. 2A). This mRNA encodes the mature Lb<sup>pro</sup> sequence followed by VP4 and 23 amino acids of VP2. The Lb<sup>pro</sup> processes itself from the growing polypeptide chain by cleavage between its own C-terminus

and the N-terminus of VP4 (Fig. 2A). The mRNA concentration was adjusted so that the rate of protein synthesis was as close as possible to that in Fig. 1 (compare Fig. 1A with Fig. 2B, middle panel). Two further mRNA concentrations were also included to investigate whether the cleavage effi-

Table 1  
Efficiency of eIF4G cleavage by Lb<sup>pro</sup> and 2A<sup>pro</sup>

RNA	50% eIF4G cleavage at (min) <sup>a</sup>	Proteinase concentration at 50% eIF4G cleavage		Approximate molar ratio proteinase:eIF4G <sup>b</sup>
		pg/μl	nM	
FMDV Lb <sup>pro</sup>	4	15	0.76	1:130
FMDV Lb <sup>pro</sup> VP4VP2				
0.25×	8	14	0.71	1:130
1×	4	16	0.81	1:130
4×	≤4	≤30	≤1.52	≥1:66
HRV2 2A <sup>pro</sup>	15	137	8.4	1:12

<sup>a</sup>Similar results were obtained in five separate experiments for Lb<sup>pro</sup> and Lb<sup>pro</sup>VP4VP2 and two for 2A<sup>pro</sup>.

<sup>b</sup>The concentration of eIF4G in RRLs was estimated as 0.1 μM, using data from Rau et al. [23] and Borman et al. [27].

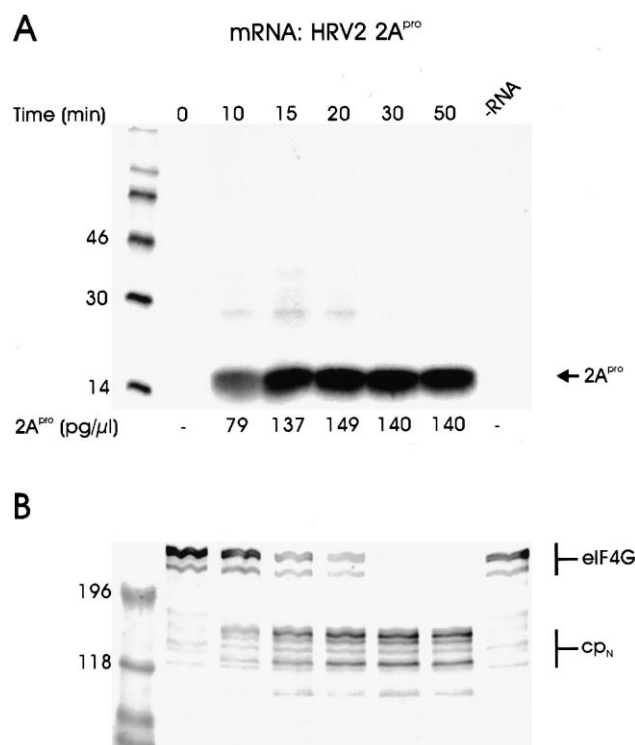


Fig. 3. Kinetics of eIF4G cleavage during in vitro translation of HRV2 2A<sup>pro</sup>. Protein synthesis from the uncapped mRNA HRV2 2A<sup>pro</sup> (15 ng/μl) was as described in Fig. 1, as was the analysis of the synthesis of 2A<sup>pro</sup> (A) and the cleavage of eIF4G (B). The concentration (in pg/μl) of 2A<sup>pro</sup> reached at each time point is indicated underneath the lane.

ciency was affected by the rate of translation (Fig. 2B, left and right panels).

Synthesis of the mature Lb<sup>pro</sup> was detectable after 4 min in all three reactions and continued to rise over the periods investigated (Fig. 2B). As the amount of uncleaved L<sup>pro</sup>VP4VP2 remained very low, even at later time points, self-processing was very efficient. The rate of Lb<sup>pro</sup> synthesis increased with increasing concentration of the mRNA. Nevertheless, in each experiment, 50% of cleavage of eIF4G had occurred when a concentration of 14–30 pg/μl Lb<sup>pro</sup> had been reached (Fig. 2C). This corresponds to the 8 min time point in the lowest mRNA concentration, and the 4 min time points for the two higher mRNA concentrations (Fig. 2C). In contrast, when protein synthesis was initiated with the mutant mRNA FMDV Lb<sup>pro</sup>C51A VP4VP2, neither processing of the Lb<sup>pro</sup> from the VP4VP2 protein nor cleavage from eIF4G was observed, demonstrating that the observed proteolytic reactions were dependent on an active Lb<sup>pro</sup>.

Table 1 summarises the results from both experiments and gives the molar ratios of proteinase to eIF4G. The eIF4G concentration in RRLs was estimated from data from Rau et al. [23] and Borman et al. [27] to be 0.1 μM. Clearly, there is no difference in the cleavage of eIF4G when the RRLs were programmed to synthesise the same amounts of Lb<sup>pro</sup> or Lb<sup>pro</sup>VP4VP2 (Table 1, compare Lb<sup>pro</sup> with Lb<sup>pro</sup>VP4VP2, mRNA concentration 1×). Thus, efficient cleavage of eIF4G by the Lb<sup>pro</sup> occurs whether or not the proteinase must process itself from the growing polypeptide chain. In addition, the same kinetics were also obtained when the translation was driven by the EMCV internal ribosome entry site

(data not shown), indicating that the reaction is not dependent on the mode of protein synthesis initiation.

### 3.3. Cleavage of eIF4G during protein synthesis of HRV2 2A<sup>pro</sup>

eIF4G is also processed during the replication of human entero- and rhinoviruses by the proteinase designated 2A<sup>pro</sup>. To investigate whether the cleavage of eIF4G by 2A<sup>pro</sup> during in vitro protein synthesis had similar kinetics to cleavage by the FMDV Lb<sup>pro</sup>, RRLs were programmed with the mRNA HRV2 2A<sup>pro</sup>, which encodes the mature proteinase followed by two stop codons. Protein synthesis and the state of eIF4G were examined (Fig. 3A,B). The eIF4G cleavage kinetics of HRV2 2A<sup>pro</sup> are different from those of Lb<sup>pro</sup>, even though the rate of synthesis of the two proteinases was similar (compare Fig. 1A, 12 min time point and Fig. 3A, 10 min time point). After 10 min, only about 20% of the eIF4G had been cleaved, although the concentration of HRV2 2A<sup>pro</sup> had reached 79 pg/μl (4.85 nM). 50% cleavage was obtained after 15 min (Fig. 3B), when the concentration of 2A<sup>pro</sup> was 137 pg/μl (8.4 nM). This represents a molar ratio of 2A<sup>pro</sup> to eIF4G of 1:12 (Table 1).

## 4. Discussion

The data presented here show that the cleavage of eIF4G during synthesis of FMDV Lb<sup>pro</sup> and HRV2 2A<sup>pro</sup> can be extremely efficient. The reaction occurs rapidly at low molar ratios of proteinase to eIF4G, similar to those calculated to be found during in vivo replication [13]. In addition, the rate of the reaction is higher and the molar ratios of proteinase to eIF4G are much lower than those found when pure preparations of proteinases are incubated with purified preparations of eIF4G, eIF4G and eIF3, or when HeLa cell extracts are used as a source of eIF4F [12,13,16,17]. For example, in experiments using purified poliovirus 2A<sup>pro</sup> and a purified complex of eIF4F/eIF3, a molar ratio of proteinase to eIF4G of 2.5:1 was required to obtain 50% cleavage of eIF4G over a 2 h period [13].

Two differences are apparent in the kinetics of cleavage between the two proteinases. Firstly, the Lb<sup>pro</sup> concentration required for 50% eIF4G cleavage is significantly lower than that required by 2A<sup>pro</sup>. Secondly, the onset of cleavage by Lb<sup>pro</sup> is more rapid. This suggests that Lb<sup>pro</sup> folds and becomes active either during or immediately after its synthesis, whereas 2A<sup>pro</sup> requires some time to reach its active state. As 2A<sup>pro</sup> needs a zinc ion for its structural integrity, the enzyme may require longer to fold correctly [31,32]. These differences may explain why in vivo cleavage of eIF4G during FMDV replication occurs earlier (1–1.5 h post infection (p.i.)) than in enteroviruses (2 and 3 h p.i. for poliovirus and coxsackievirus, respectively) and HRVs (3–4 h p.i.) [13,16].

Why is the eIF4G cleaved so efficiently in this system? Firstly, in actively translating ribosomes in RRLs, most of the eIF4G has been shown to be complexed with eIF4E and with the ribosomes [23]. As the complex of eIF4G and eIF4E has been demonstrated to be a better substrate for HRV2 2A<sup>pro</sup> than eIF4G itself [17], this could promote eIF4G cleavage. Secondly, the synthesis of a viral proteinase on the ribosome brings it into close proximity with ribosome bound eIF4G, possibly increasing the interaction between enzyme and substrate, and thus further enhancing eIF4G cleavage. For a picornaviral infection, therefore, this implies that the

cleavage of eIF4G can occur during the initial phase of protein synthesis from the newly replicated viral RNA, before detectable amounts of the viral proteins are present.

**Acknowledgements:** This work was supported by the Austrian Science Foundation (P-11122 and P-13667 to T.S.). We thank Joachim Seipelt and Dieter Blaas for critical reading.

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